ApoE gene therapy could have therapeutic value in another apoE-related lipid disorder sometimes called familial dysbetalipoproteinemia or Type III hyperlipoproteinemia [40]. In this disorder, a mutant apoE has decreased binding to receptors, thus resulting in a reduced clearance of remnant lipoproteins and hyperlipidemia. The most common cause is homozygosity for a common polymorphism in apoE called apoE2 (the wild-type form is known as apoE3). Rarer causes are other apoE mutations that often cause hyperlipidemia in a dominant fashion. Gene therapy to increase the hepatic production of wild-type apoE3 could be a therapeutic strategy for this condition.

Familial combined hyperlipidemia and diabetic dyslipidemia

Familial combined hyperlipidemia is an inherited phenotype characterized by elevated VLDL and LDL; it is believed to be normally due to overproduction of VLDL by the liver [41]. The specific genetic basis of this common disorder is currently unknown. Type II diabetes mellitus is also characterized by the hepatic overproduction of VLDL and increased VLDL and LDL cholesterol [42]. Both of these conditions are associated with a significantly increased risk for atherosclerotic vascular disease. Although many patients with these disorders can be adequately treated with lifestyle and drug therapy, a substantial number cannot achieve adequate control of their hyperlipidemia. Gene therapy might therefore be one therapeutic approach to this class of patients with refractory combined hyperlipidemia.

One approach could be expression of the VLDL receptor in the liver. As noted above, although the VLDL receptor is not normally expressed in the liver, it could theoretically bind VLDL and reduce the levels of both VLDL and LDL. Proof-of-principle studies in relevant animal models are needed to address this question. Another approach could be increasing the hepatic expression of apoE. Because apoE serves as a ligand for the LDL receptor, other related receptors, and heparan sulfate proteogycans in the liver, increased apoE expression could potentially target more VLDL and remnants for hepatic degradation, thus reducing VLDL and LDL levels. Again, proof-of-principle studies in relevant animal models are needed to address this question. Specifically, more data are needed on the effects of hepatic apoE gene transfer on lipoprotein metabolism in dyslipidemic animal models other than apoE deficiency.

Disorders associated primarily with low-HDL cholesterol

Lecithin : cholesterol acyltransferase deficiency

Unesterified cholesterol is esterified to cholesteryl ester in the blood by the lipoprotein-associated enzyme lecithin : cholesterol acyltransferase (LCAT). Complete LCAT deficiency is is characterized by markedly reduced HDL cholesterol levels (less than 10 mg/dl), corneal opacities, anemia, and progressive proteinuria and renal insufficiency eventually leading to end-stage renal disease [43]. Several different mutations in the LCAT gene have been described in patients with LCAT deficiency. LCAT knockout mice also have reduced HDL cholesterol levels [44], although the development of renal disease has not been reported. Because this disorder has no known therapy to prevent the progressive renal disease, it is a candidate for the development of gene therapy. Although LCAT is normally synthesized in the liver, because it is a secreted protein it could theoretically be made in other tissues such as muscle after gene transfer.

Tangier disease ✱

Tangier disease is a rare genetic disorder associated with markedly reduced HDL cholesterol levels (less than 5 mg/dl), the accumulation of cholesterol in macrophages and related cells, neuropathy, and premature atherosclerosis [45]. It is caused by mutations in the ATP-binding cassette protein 1 (ABC1), a cellular protein that promotes the efflux of cellular cholesterol and phospholipids to nascent HDL and lipid-poor apolipoprotein A-I (apoA-I) [46]. ABC1 knockout mice also have markedly reduced HDL cholesterol levels and cholesterol storage in macrophages and macrophage-like cells [47]. A naturally occurring model of Tangier disease in chickens has been reported [48]. Gene transfer of ABC1 to macrophages or bone marrow stem cells would be likely to ameliorate the symptoms of this disorder, but no proof-of-principle studies have yet been reported in animals.

Importantly, heterozygotes for ABC1 mutations have significantly reduced HDL cholesterol levels (less than 10th percentile) [49] and are at increased risk for atherosclerotic vascular disease [50]. There are no drug therapies that raise HDL cholesterol levels in these patients. Macrophage-directed gene therapy with ABC1 could therefore be an approach to this disorder as well.

ApoA-I deficiency

ApoA-I is the major protein in HDL; a genetic deficiency of apoA-I results in markedly reduced HDL and seems, at least in some kindreds, to increase the risk for coronary artery disease [51]. ApoA-I knockout mice have extremely low levels of HDL cholesterol and although chow-fed mice do not develop atherosclerosis [52], the absence of apoA-I enhances the progression of atherosclerosis in hyperlipidemic mouse models [53,54]. The expression of human apoA-I in apoA-I knockout mice by using a first-generation adenovirus transiently restored HDL cholesterol levels to the normal range [55]. In apoA-I-deficient patients, gene transfer of apoA-I to liver (or possibly muscle) would be expected to increase HDL cholesterol levels and over time would reduce the risk for atherosclerotic disease. However, apoA-I-deficient patients are very rare, making this disease somewhat less attractive as a target for the development of gene therapy.

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 805–810, February 1999
Biochemistry

Niemann-Pick C1 protein: Obligatory roles for N-terminal domains and lysosomal targeting in cholesterol mobilization

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Contributed by Roscoe O. Brady, November 25, 1998

ABSTRACT **Niemann-Pick type C (NPC) disease is an inherited lipid storage disorder that affects the viscera and central nervous system. A characteristic feature of NPC cells is the lysosomal accumulation of low density lipoprotein-derived cholesterol. To elucidate important structural features of the recently identified *NPC1* gene product defective in NPC disease, we examined the ability of wild-type NPC1 and NPC1 mutants to correct the excessive lysosomal storage of low density lipoprotein-derived cholesterol in a model cell line displaying the NPC cholesterol-trafficking defect (CT60 Chinese hamster ovary cells). CT60 cells transfected with human wild-type NPC1 contained immunoreactive proteins of 170 and 190 kDa localized to the lysosomal/endosomal compartment. Wild-type NPC1 protein corrected the NPC cholesterol-trafficking defect in the CT60 cells. Mutation of conserved cysteine residues in the NPC1 N terminus to serine residues resulted in proteins targeted to lysosomal membranes encircling cholesterol-laden cores, whereas deletion of the C-terminal 4-aa residues containing the LLNF lysosome-targeting motif resulted in the expression of protein localized to the endoplasmic reticulum. None of these mutant NPC1 proteins corrected the NPC cholesterol-trafficking defect in CT60 cells. We conclude that transport of the NPC1 protein to the cholesterol-laden lysosomal compartment is essential for expression of its biological activity and that domains in the N terminus of the NPC1 protein are critical for mobilization of cholesterol from lysosomes.**

Niemann-Pick type C (NPC) disease is an autosomal recessive neurovisceral lipid storage disorder (1, 2). One of the most pronounced abnormalities in affected cells is the accumulation of free cholesterol derived from low density lipoproteins (LDL) in lysosomes and the Golgi apparatus (3–7). This biochemical phenotype is displayed by cells from NPC patients (3–7), cells from mice homozygous for the spontaneously occurring C57BLKS/J*spm* and BALB/c *npcnih* mutations (8), in addition to Chinese hamster ovary cell mutants generated in the laboratory, including the CT60 line (9–12).

A gene that is mutated in human NPC disease, named *NPC1*, recently was identified by positional cloning (13). This nomenclature recognizes that mutations in at least one other distinct gene can cause the disorder. The human *NPC1* cDNA sequence was isolated from a yeast artificial chromosome that corrected the cholesterol-trafficking defect in human NPC fibroblasts, cells derived from mice homozygous for the BALB/c *npcnih* mutation, and CT60 cells (13, 14). The gene mutated in BALB/c *npcnih* mice was shown to be the murine homolog of the human *NPC1* gene (15).

The human *NPC1* cDNA sequence predicts a protein of 1,278 aa with an estimated molecular mass of 142 kDa (13). A region between amino acid residues 55 and 165, which is free of transmembrane domains, is highly conserved between the human and murine *NPC1* sequences as well as orthologs in *Caenorhabditis elegans* and *Saccharomyces cervisiae* (13, 15). This NPC domain, consisting of 112 aa, contains eight cysteine residues with conserved spacing in all *NPC1* orthologs. Beyond this N-terminal sequence lies up to 16 putative transmembrane domains and a sequence with strong homology to the sterol-sensing domains of 3-hydroxy-3-methylglutaryl CoA reductase, the sterol response element binding protein cleavage activating protein (SCAP), and the Hedgehog signaling protein, Patched (13, 15). The C terminus of NPC1 contains a dileucine motif that serves as a lysosomal targeting sequence for a lysosomal integrated membrane protein, LIMPII (16–18). In the present study, we undertook experiments to identify structural features of the human NPC1 protein that are critical for its actions on intracellular cholesterol trafficking.

MATERIALS AND METHODS

***NPC1* cDNA Expression Constructs.** To produce the template plasmid DNA for site-directed mutagenesis, wild-type *NPC1* cDNA (13) was subcloned into the pSV-SPORT-1 vector (GIBCO). The C63S, C74S/C75S, and C97S mutations and the deletion of the C-terminal 4-aa residues (C-4) were produced by site-directed mutagenesis using reagents purchased from CLONTECH. Each construct was sequenced to confirm that the desired mutation(s) had been introduced into the cDNA as described (19).

Cell Culture and Transfection. The Chinese hamster ovary CT60 cells were generously provided by T. Y. Chang (Dartmouth University, Hanover, NH). The cells were cultured as described (11). In other studies, a line of H-ras and temperature-dependent simian virus 40 large T antigen-transformed granulosa cells derived from BALB/c *npcnih* were studied (14). These cells display the characteristic NPC sterol-trafficking defect.

For transfection, cells were cultured in 6-well plastic culture plate to 50–80% confluence. CT60 cells were transfected by using LipofectAMINE PLUS reagent (Life Technologies, Grand Island, NY) with 2.0 μg/ml of either an empty pSV-SPORT-1 vector, the wild-type, or the mutant *NPC1* cDNAs in pSV-SPORT-1 with or without 0.2 μg/ml of the plasmid expressing an enhanced green fluorescent protein (EGFP) mutant carrying a nuclear targeting sequence (pEGFP). pEGFP was used to identify transfected cells for analysis of

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Abbreviations: NPC, Niemann-Pick type C; LDL, low density lipoprotein; EGFP, enhanced green fluorescent protein.

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filipin staining. The media were changed to the regular growth media or to media supplemented with 10% lipoprotein-deficient serum for Western blot analysis and for filipin staining and immunocytochemistry, respectively, after 24 h.

Western Blotting. Cells were scraped from the dishes into lysis buffer consisting of 100 mM Tris·HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.02% sodium azide, 100 μg/ml of phenylmethylsulfonyl fluoride, and 1 μg/ml of aprotinin and centrifuged at 10,000 × *g* for 2 min at 4°C, and supernatants were subjected to SDS/PAGE and then Western blotting (19). A polyclonal antibody (NPC1-C antibody) raised against a peptide corresponding to 19-aa residues (NKAKSCATEERYKGTERER) in the C terminus of human NPC1 (amino acid residues 1256–1274) was used to detect NPC1 (S.P., unpublished work).

Filipin Staining. Transiently transfected cells were fixed with 3% paraformaldehyde in PBS for 30 min. Cells were washed in PBS (three times for 5 min), quenched with 1.5 mg/ml glycine in PBS for 10 min, and stained with 0.05 mg/ml of filipin (Polysciences) in PBS for 30 min. After washing in PBS (three times for 5 min), slides were mounted with coverslips in phenylenediamine/glycerol. For quantitative analysis, 100 EGFP positive cells were selected and viewed with 25× objective. Intense filipin fluorescence staining of cholesterol in large perinuclear granules is characteristic of the lysosomal cholesterol accumulation in NPC cells (3–5, 13, 14). Cells with markedly reduced filipin-fluorescence were scored as corrected. The corrected cells contained fewer and smaller filipin-positive granules. Cells with some larger filipin-positive granules, representing an intermediate pattern between uncorrected and corrected were scored as uncorrected in our analysis. Values presented are means ± SE from three separate experiments. ANOVA and the Tukey-Kramer test were used to determine significant differences between empty vector control and wild type or mutants. $P < 0.05$ was used as the level of significance.

Immunocytochemical Staining of NPC1. CT60 cells grown on uncoated glass coverslips were transfected as described above. At the end of the culture period, cells were fixed with 3% paraformaldehyde in PBS for 30 min. Cells were immunostained with an indirect procedure as described (6). Immunostaining for NPC1 was done with rabbit affinity purified anti-peptide NPC1-C antibodies and for lysosomal membrane glycoprotein, mouse monoclonal anti-lgp95 (20). Secondary, fluorescently labeled goat anti-rabbit and anti-mouse IgG was from Jackson Immunoresearch. Stained cells were examined with a Zeiss LSM 410, confocal microscope equipped with a UV laser.

RESULTS

Wild-Type NPC1 Is Transported to Lysosomes and Corrects the NPC Cholesterol-Trafficking Defect. To investigate the biological function of wild-type NPC1, we transfected CT60 cells with expression plasmids and performed filipin staining to detect free cholesterol in the lysosomal compartment. The cholesterol-loaded granules were identified as lysosomes based on previous studies of normal and NPC fibroblasts that established that the mass of endocytically derived cholesterol is accumulated in lysosomes during the first 24 h of incubation

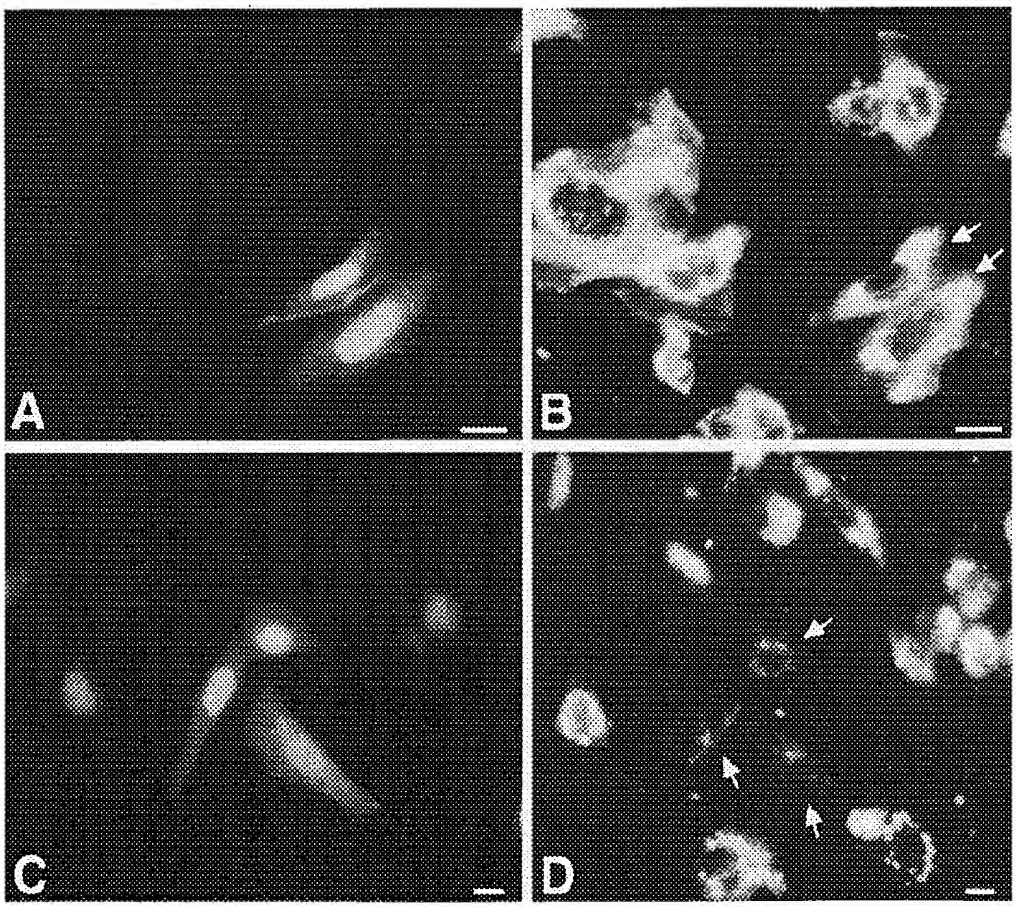

FIG. 1. Photomicrographs of filipin-stained CT60 cells transfected with empty vector or wild-type NPC1 expression plasmid. (*A*) EGFP expression by CT60 cells transfected with pEGFP and empty vector. (*B*) Filipin staining. Arrows indicate EGFP-expressing cells. (*C*) EGFP expression by CT60 cells transfected with pEGFP and wild-type NPC1 expression plasmid. (*D*) Filipin staining. Arrows indicate EGFP-expressing cells. (Bar = 10 μm.)

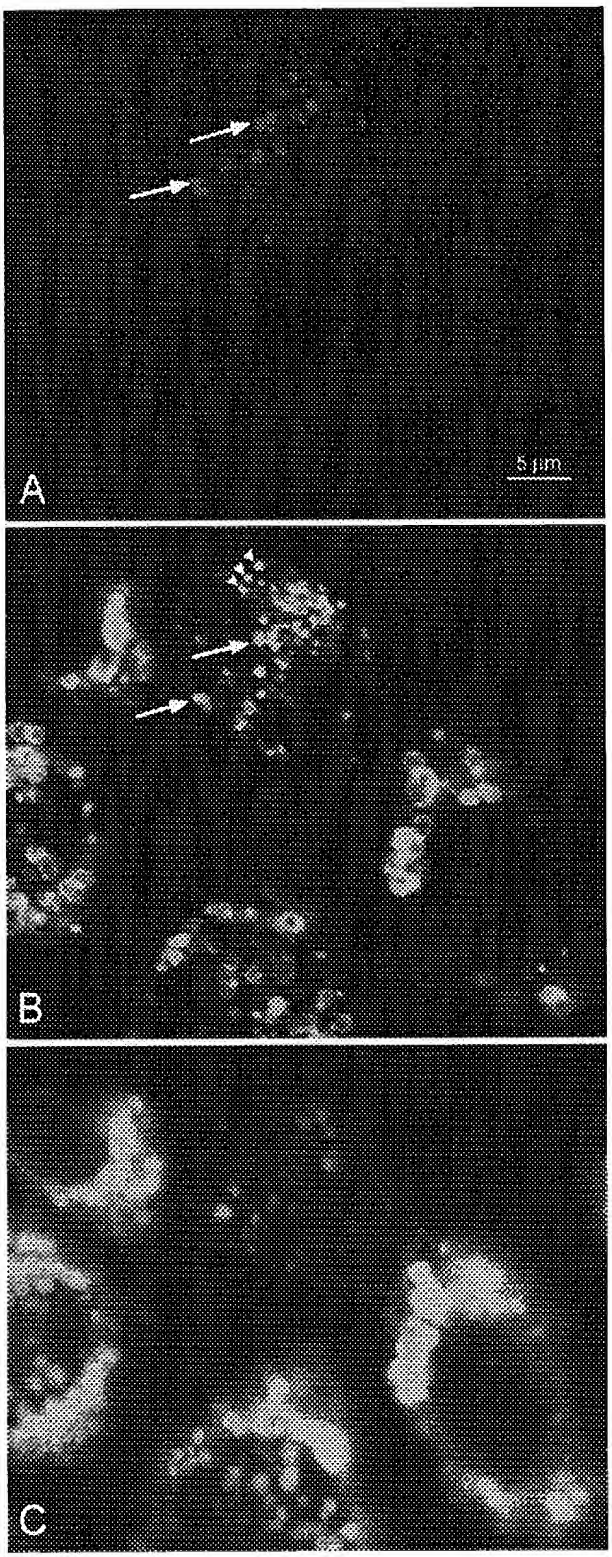

FIG. 2. CT60 cell transfected with wild-type NPC1 immunostained for (*A*) NPC1 protein and (*B*) lysosomal membrane glycoprotein (lgp95) and (*C*) stained with filipin for cholesterol localization. (*A*) NPC1 is present in the transfected cell in granular structures that colocalize with lgp95 (in *B* at arrows). There are additional lgp95 containing organelles (in *B* at arrowheads) that do not contain NPC1 protein. Thus NPC1 protein is in a subset of the lgp95-containing organelles. (*C*) The transfected CT60 cell expressing NPC1 protein does not contain accumulations of intracellular cholesterol in lgp95 containing lysosomes as seen in the four adjacent cells not expressing NPC1 protein. (Bar = 5 μm.)

with LDL. We used pEGFP to identify the transiently transfected cells. EGFP-positive CT60 cells transfected with empty vector and cultured in the presence of LDL showed intense filipin staining in perinuclear granules reflective of lysosomal cholesterol accumulation (Fig. 1 *A* and *B*), whereas EGFP-positive CT60 cells transfected with wild-type NPC1 typically had many fewer and smaller filipin-stained granules, implying correction of the NPC cholesterol-trafficking defect (Figs. 1 *C* and *D* and 2, and Table 1). Sixty-seven percent of the CT60 cells transfected with wild-type NPC1 protein were corrected, whereas less than 8% of the CT60 cells transfected with empty vector showed reduced filipin staining. Because partially corrected cells were scored as uncorrected in our analysis, the 67% correction rate undoubtedly underestimates the impact of NPC1 expression in the CT60 cells.

We also transfected a line of granulosa cells derived from the BALB/c *npcnih* mice with the human NPC1 expression plasmid and found that transfected cells had reduced filipin staining, whereas cells transfected with empty vector did not (data not shown). Because the transfection efficiency of the granulosa cells was quite low, all further experiments were carried out with CT60 cells.

To assess the subcellular localization of wild-type NPC1 protein, immunocytochemical studies were carried out on CT60 cells transfected with empty vector or wild-type NPC1 expression vector. The antibody used for these studies recognized human NPC1 protein in Western blots of extracts of CT60 cells transfected with NPC1 expression plasmid, but CT60 cells transfected with empty vector contained no detectable NPC1 protein (Fig. 3). CT60 cells transfected with wild-type NPC1 displayed a granular pattern of NPC1 immunostaining in 1.0-μm vesicles that were also positive for lysosomal membrane glycoprotein (lgp95), establishing that the NPC1-positive organelles were components of the endosomal/lysosomal pathway (Fig. 2). However, the NPC1 protein was present in some, but not all, of the lgp95-positive vesicles. Cells containing large filipin-positive granules did not stain for NPC1 protein, but did contain lgp95-positive vesicles (Fig. 2). Preimmune serum gave no staining of CT60 cells transfected with wild-type NPC1 expression plasmid and CT60 cells transfected with empty vector did not show NPC1 immunostaining when reacted with immune serum (data reviewed but not presented).

Mutant NPC1 Proteins That Do Not Localize to the Core of Cholesterol-Loaded Lysosomes Do Not Correct the NPC Cholesterol-Trafficking Defect. To elucidate the structural features of human NPC1 protein that are required for its subcellular localization and biological function, we introduced point mutation(s) into the human *NPC1* cDNA coding sequence and analyzed the subcellular localization and biological activities of the resulting mutant proteins. We focused attention on the N

Table 1. Correction of the NPC sterol trafficking defect in CT60 cells by expression of NPC1

Transfected plasmid	% Corrected cells
Empty vector	7.9 ± 1.0
Wild-type NPC1	67.8 ± 4.6*
C63S	8.1 ± 1.8
C74S/C75S	5.5 ± 1.0
C97S	8.3 ± 2.9
C-4	7.5 ± 1.0

CT60 cells were transfected with the indicated plasmid and the pEGFP expression plasmid. Cells were incubated with LDL and then fixed for filipin staining as described in the text. One hundred EGFP-stained cells were analyzed for filipin staining in each treatment. The values presented are the percentage of EGFP-expressing cells showing a marked reduction in perinuclear filipin-positive granules. Values are means ±SE from three separate experiments.
*Different from all other groups, $P < 0.001$.

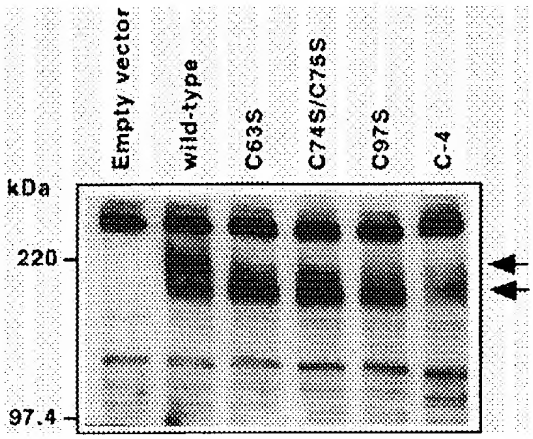

FIG. 3. Expression of wild-type NPC1 and NPC1 mutants in CT60 cells. CT60 cells were transfected with the indicated plasmids, and Western blot analysis was performed on cell extracts as described in *Materials and Methods*. Each lane contained 100 μg of protein. Arrows indicate the NPC1 bands.

and C termini of the NPC1 protein because the N terminus is highly conserved, suggesting functional importance, and the C terminus contains the dileucine motif (LLNF), which is a potential lysosomal targeting sequence. We mutated conserved cysteine residues in the N terminus (C63, C74/C75, and C97) to serine residues and introduced a stop codon (TGA) just before the dileucine motif (C-4) to delete the putative lysosomal targeting sequence.

Each of the mutant proteins was detectable in the transfected CT60 cells by Western blot analysis (Fig. 3). However, in contrast to the wild-type NPC1 protein that yielded proteins of 170 and 190 kDa, the mutant NPC1 expression plasmids each yielded a major immunoreactive protein of 170 kDa. These findings were confirmed with a polyclonal antiserum generated against a recombinant protein representing amino acid residues 25–266 of the human NPC1 N terminus (data not shown).

Immunocytochemical studies revealed that N-terminal mutant proteins, C63S, C74S/C75S, and C97S, were localized at the surface of lysosomes. Fig. 4 shows representative immunostaining of the C97S mutant. These NPC1 domain mutant proteins were visualized as rings (Fig. 4*B*) surrounding filipin-stained spheres (Fig. 4 *A* and *D*). The mutant NPC1 protein (Fig. 4*B*) colocalized with lgp95 (Fig. 4*C*), indicating that it was restricted to the membrane surface of lysosomes whose cores were loaded with free cholesterol. A similar pattern of immunostaining was obtained with the C63S and C74S/C75S mutants (data not shown).

The C-4 mutant lacking the lysosomal targeting sequence was localized to intracellular membranes displaying a reticular pattern characteristic of the endoplasmic reticulum and in the nuclear envelope (Fig. 5 *A* and *B*). None of the N-terminal mutant NPC1 proteins or the C-4 mutant lacking the dileucine motif corrected the cholesterol-trafficking defect in the CT60 cells identified by filipin staining (Table 1).

DISCUSSION

In the present study we have shown that wild-type NPC1 protein is localized in a subset of vesicles containing lgp95, a marker for both late endosomes and lysosomes in Chinese hamster ovary cells (20). In human fibroblasts, we found a similar localization of NPC1 protein in a subset of vesicles containing LAMP2 (21), a marker for late endosomes and lysosomes (22). The LAMP2-positive, NPC1-containing vesicles, linked to a prelysosomal compartment in fibroblasts by kinetic studies on uptake of [^{14}C] sucrose, are distinct from LAMP2-positive lysosomes that accumulate cholesterol derived from LDL. The fibroblast study also suggested that wild-type NPC1-containing vesicles interact transiently with the cholesterol-laden lysosomes, and that under certain experimental conditions, NPC1 protein can enter the core of these organelles (21). Thus, NPC1 appears to move through prelysosomal vesicles before its entry into the lysosomal compartment. In the CT60 cells transfected with wild-type NPC1 expression vector, the lgp95-positive, NPC1-containing vesicles may be prelysosomal vesicles, similar to those that we

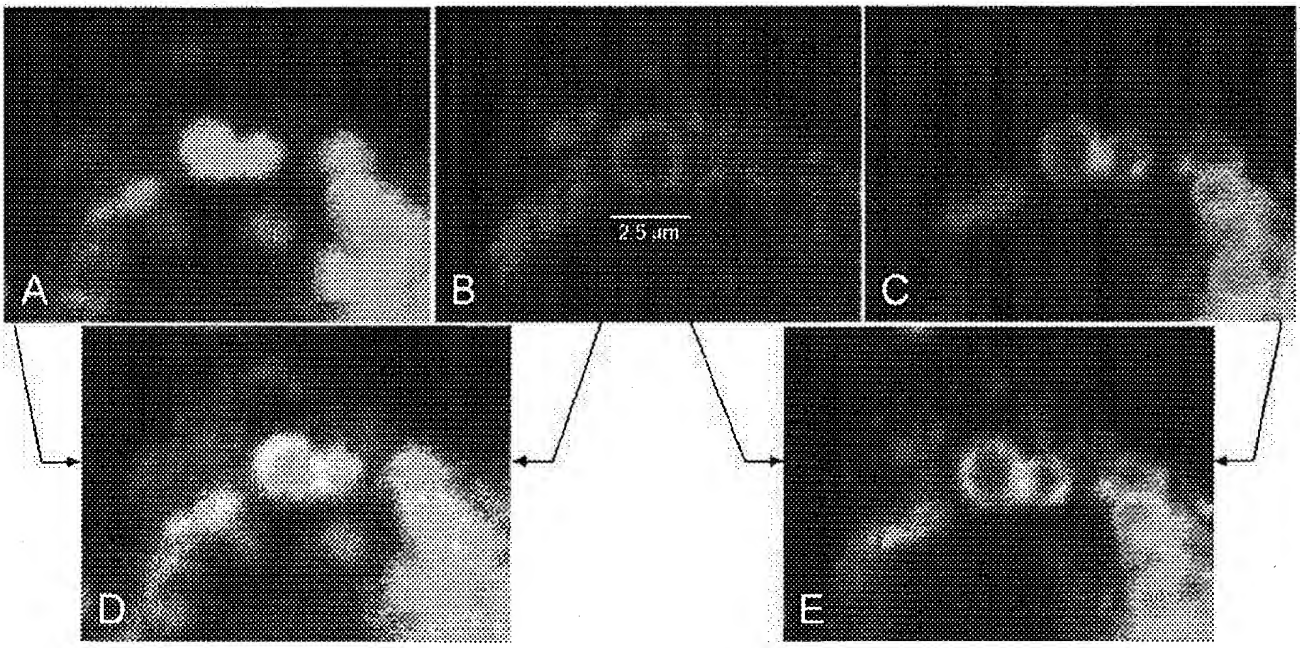

FIG. 4. CT60 cell transfected with the C97S NPC1 mutant. (*A*) Filipin staining showed transfected cells did not clear of cholesterol and that the NPC1 protein (*B*) was present in rings at the surface of the cholesterol-laden cores of lysosomes that are lgp95 positive (*C*). The lgp95 immunostaining that marks protein in the lysosomal membrane colocalizes with the mutant NPC1 protein, indicating that the mutant NPC1 is at the surface of lysosomes. *D* is the merged image of *A* and *B*; and *E* is the merged image of *B* and *C*. (Bar = 2.5 μm.)

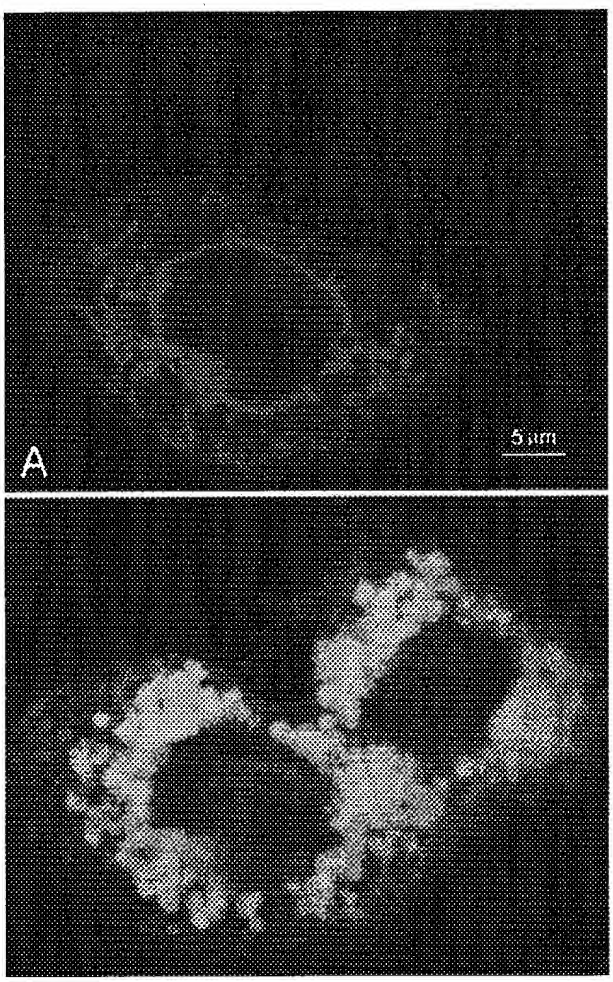

FIG. 5. CT60 cell transfected with the C-4 NPC1 mutant lacking the dileucine motif. The mutant NPC1 protein (*A*) is present in a reticular pattern characteristic of the endoplasmic reticulum and in the nuclear envelope (*B*). The cell expressing mutant NPC1 has not cleared of cholesterol evidenced by its accumulation of filipin-positive lysosomes similar to the adjacent cell, not expressing mutant NPC1 protein. (Bar = 5 μm.)

observed in fibroblasts, or they may represent lysosomes that have been cleared of cholesterol. In contrast, the nonfunctional N-terminal mutant NPC1 proteins appear to be retained on the surface of the cholesterol-laden lysosomes.

The NPC1 C terminus contains a dileucine motif (LLNF). Related dileucine motifs mediate lysosomal targeting and internalization of proteins from the cell surface (16–18). Deletion of the dileucine motif from the NPC1 C terminus resulted in a protein that accumulated in the endoplasmic reticulum and was incapable of correcting the NPC sterol trafficking abnormality. The N terminus of NPC1 contains 13 hydrophobic amino acid residues typical of signal peptides that target proteins to the endoplasmic reticulum. Thus, newly synthesized NPC1 protein may be directed to the endoplasmic reticulum first, and then sorted through a prelysosomal compartment and ultimately to lysosomes by the dileucine motif. The C-terminal mutant lacking the lysosomal targeting dileucine motif may not be able to escape from the endoplasmic reticulum, accounting for the immunocytochemical distribution that we observed with the C-4 mutant. It is also possible that the NPC1 dileucine motif is essential for protein function once NPC1 enters the lysosomal compartment. However, this possibility cannot be evaluated in our experimental system.

Mutations in the conserved cysteine residues in the NPC1 domain also caused loss of biological activity despite targeting of the mutant proteins to the surface membranes of lysosomes. This localization to membranes encircling cholesterol-loaded spheres raises the possibility that NPC1 must enter into the lysosome core, which is compacted with cholesterol-enriched multilamellar structures (23), to function. This notion is consistent with our observations on human fibroblasts where NPC1 could be found in the core of cholesterol-laden lysosomes (21). That all three N-terminal cysteine residue mutants lost biological activity suggests that the NPC1 domain is functionally important. The correct formation of intra- or interchain disulfide bonds may be crucial for the proper assembly of NPC1 proteins, which in turn could influence maintenance of an active confirmation. A misfolded N-terminal domain might prevent movement of the NPC1 protein to its site of action within the lysosomal core.

The human *NPC1* cDNA sequence predicts a protein with an estimated molecular mass of 142 kDa. However, two proteins of larger size, 170 and 190 kDa, were detected in cells transfected with the wild-type NPC1 expression plasmid. These immunoreactive proteins presumably represent post-translationally modified NPC1. There are 14 potential N-glycosylation sites conserved in human and mouse NPC1 (13, 15). In studies to be reported elsewhere, we found that treatment of extracts of COS-1 cells transfected with NPC1 expression plasmid with *N*-glycosidase F resulted in an 18-kDa reduction in the apparent molecular mass of a tryptic peptide recognized by a polyclonal antiserum raised against residues 25–266 of the NPC1 N terminus (H.W. and J.F.S., unpublished observations). This finding confirms that NPC1 is glycosylated. Interestingly, all of the biologically inactive mutant constructs yielded a major 170-kDa product in the CT60 cells, suggesting that a posttranslational modification resulting in the formation of the 190-kDa immunoreactive species may be essential for NPC1's functional activity, or alternatively, that the NPC defect influences protein glycosylation. The latter idea is consonant with the recent observations of Nohturfft *et al.* (24) revealing sterol regulation of the processing of the carbohydrate chains of SCAP, a protein that shares structural features with NPC1.

Although we examined the ability of wild-type NPC1 and NPC1 mutants to correct the excessive lysosomal accumulation of LDL-derived free cholesterol in the present studies, it also is known that NPC cells show enrichment of cholesterol in trans-cisternal Golgi compartments and delayed relocation of cholesterol to and from the plasma membrane (6, 25). Further studies are needed to determine whether the wild-type and mutant NPC1 proteins can correct these alterations in intracellular cholesterol movement. Such studies could provide important insight into the complexities of intracellular cholesterol transport (26–29).

We thank Ms. Judith Wood for help in preparation of this manuscript and Dr. T. Y. Chang for the gift of CT60 cells. This work was supported by National Institutes of Health Grants HD06274 (J.F.S.) and NS34339 (S.P.) and a grant from the Ara Parseghian Medical Research Foundation.

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Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 10950–10952, September 1999

Commentary

Effluxed lipids: Tangier Island's latest export

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In 1608, the intrepid English explorer, Captain John Smith, set out to map the new colony of Virginia and its Chesapeake Bay environs (1). Approximately 20 miles west of the Eastern Shore, he encountered three islands that he named the Russells after his ship's surgeon, Dr. William Russell. Today, one of the islands bears Smith's name and another is called Tangier Island because, according to local lore, its sandy shores reminded the captain of the white dunes of the port of Tangier, Morocco on the Strait of Gibraltar (2). For most of the past 400 years, the inhabitants of Tangier Island have been both economically sustained and genetically insulated by the bay that surrounds them. A calamitous outbreak of cholera in 1866 led to the evacuation and quarantine of the island, and its subsequent repopulation by a much smaller number of the displaced islanders added to the genetic insularity of its inhabitants. Most of the island's current population are descendants of this small group of hardy individuals, with more than 600 of the approximately 800 residents bearing the surname Crockett, Pruitt, or Parks (2). It is not surprising, therefore, that when another explorer, Donald Fredrickson of the National Institutes of Health, traveled to Tangier in 1960 to find individuals who shared the phenotype of orange (cholesterol ester-laden) tonsils that he and his colleagues had found in a young boy from the island, the one similar individual identified was the boy's younger sister (3). The findings of orange, lipid-engorged tonsils and nearly absent high density lipoprotein (HDL) cholesterol levels, shared by the siblings, are the hallmarks of the disorder to which the island has given its name, Tangier Disease (4).

In the past decade, studies of fibroblasts taken from patients with Tangier Disease have demonstrated a defect in the export of cholesterol from these cells to the major protein constituent of HDL, apolipoprotein AI (apoAI) (5–7). The failure of cells to efflux cholesterol to HDL *in vivo* would lead to a lipid-depleted HDL in the circulation. Metabolic turnover studies have shown that such lipid-poor HDL are very rapidly cleared from the blood, probably by the kidney (8, 9). The failure to move cellular cholesterol to HDL therefore would account, at least in part, for the low levels of HDL and HDL cholesterol seen in Tangier patients. Although these observations have delineated the general metabolic problem in patients with Tangier Disease, the cell biologic explanation for the efflux abnormality has proven more refractory to our understanding. In this issue of the *Proceedings*, Takahashi and Smith (not to be confused with the seafaring captain) (10) report a novel mechanism through which apoAI appears to remove cholesterol from cells. It is this specific process of apolipoprotein-stimulated cholesterol efflux that is most clearly defective in individuals with Tangier Disease.

Cells have evolved very elegant mechanisms for controlling their cholesterol content. Although our understanding of these mechanisms is chiefly the result of work involving the *de novo* cholesterol synthesis and receptor uptake pathways whose activation increases cellular cholesterol content, there are also pathways for ridding cells of excess cholesterol (11). As cholesterol accumulates in cells, it can be stored in its acylated form in cytoplasmic lipid droplets that arise from the action of the cholesterol-esterifying enzyme, acyl-CoA-cholesterol-acyltransferase (ACAT). Mobilization of this stored cholesterol can be stimulated by incubating cells with apoAI. The cholesterol ester is hydrolyzed to unesterified cholesterol and then traffics to the plasma membrane via a route that is not well mapped. The apolipoprotein acceptor then acquires the cholesterol in a process that entails more than simple desorption and diffusion from the plasma membrane. If the acceptor is apoAI in a nascent HDL particle, the cholesterol can be re-esterified by the action of an associated enzyme, lecithin cholesterol acyl transferase (LCAT), and then stored in the nonpolar lipid core of the HDL particle. This process triggers what often is referred to as the reverse cholesterol transport pathway and has long been postulated to represent the physiologic basis for the association of elevated HDL cholesterol levels with lower rates of coronary heart disease (12). By removing cholesterol from the cells in which it chiefly accumulates in atherosclerotic lesions (i.e., the monocyte/macrophages), the reverse cholesterol transport pathway provides a means by which the artery wall can protect itself from unwanted lipid deposition.

In previous work, Smith's laboratory showed that a mouse macrophage cell line, RAW 264, had a cAMP-inducible increase in cholesterol efflux to another lipid acceptor apolipoprotein, apoE (13). Treatment with the cAMP analogue, 8-Br-cAMP, increased the binding of both apoE and apoAI to the treated RAW cells, suggesting that increased adenylate cyclase activity led to greater expression, or conformational activation, of the plasma membrane protein responsible for tethering the apolipoproteins to the cell. As the identity of this docking protein is unknown, it is a reasonable candidate for the protein that is defective in Tangier Disease. In the current paper, the authors examined the one established HDL receptor, SR-BI (14), for cAMP responsiveness and found a substantial decrease in its expression, making it unlikely to be the protein responsible for enhanced binding. More interestingly, the authors found that cAMP treatment resulted in a dramatic increase in the internalization of radiolabeled apoAI and a subsequent resecretion of 58% of the cell-associated label. Although, SR-BI mediates lipid transfer from HDL into cells, the available evidence indicates that this receptor's activity does not result in the internalization of the protein component of the lipoprotein. Thus, it would appear that a novel protein is required for this action. The authors also present evidence that cholesterol is released from the cells at the same time the apolipoprotein re-emerges, suggesting that internalized apoAI carries the cholesterol out with it upon resecretion. Finally, using a variety of cell biologic methods, the authors provide evidence that indicates that a calcium-dependent endocytosis pathway is involved in the process. Several years ago, Schmitz *et al.* (15) described a pathway of HDL uptake and resecretion, termed retroendocytosis, that is quite similar to what Takahashi and Smith now report for the apolipoprotein component alone. Although this earlier observation concerning HDL was controversial (16), the technical difficulties of performing

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these experiments may account for the discrepant results. The current work strengthens the evidence that the uptake of HDL or its apolipoprotein component may be necessary for efficient cholesterol efflux. Nevertheless, there are some caveats that must be mentioned in the interpretation of these studies.

The work by Takahashi and Smith was done by using a transformed mouse monocyte cell line. The actual quantitative impact of resecretion on total cholesterol efflux from these cells is difficult to assess in the data presented. In most laboratories, including our own, cholesterol efflux from lipid-loaded human fibroblasts typically represents 10–20% of the labeled cellular sterol, an amount that may be substantially higher than that arising from the resecretion pathway in RAW cells. As human fibroblasts do not require cAMP treatment to export this amount of cholesterol to apolipoprotein acceptors, it is not clear if the RAW response to cAMP involves stimulation of a specific mechanism that is common to the fibroblast and macrophage efflux pathways, or if some more general effect on macrophage function accounts for the change. Finally, some of Takahashi and Smith's data implicating endocytosis in the efflux pathway depend on chemical inhibitors whose effects may not be confined to the endocytotic events they postulate to be involved in apoAI uptake and resecretion. Despite these caveats, the data provides an intriguing insight into cholesterol efflux and the mechanism by which apoAI may stimulate it. Clearly, the identification of the protein responsible for mediating the enhanced apoAI binding that results from cAMP stimulation would seem to be the logical next step in the characterization of this pathway. Remarkably, that may have already occurred.

Over the summer, several laboratories independently identified and three groups have now published studies demonstrating that an ATP binding cassette (ABC) transporter is mutated in patients with Tangier Disease (17–19). This ABC1 transporter, originally cloned by Chimini and colleagues (20, 21) using PCR amplification based on homology to other ABC proteins, is a widely expressed, putative 12-membrane spanning protein, whose activity in macrophages is up-regulated by sterol loading. The protein adds to the growing list of ABC family members linked to human diseases, several of which involve errors in lipid handling (22–24). The papers linking this protein to Tangier Disease detail the localization of the gene within a previously mapped region of chromosome 9 (25). These reports also describe multiple mutations, several of which would clearly result in a nonfunctional protein. The studies do not, however, contain functional data that indicate that apoAI directly binds to the ABC1 transporter. So, it is by no means certain that the binding protein that Smith's laboratory finds to be up-regulated by cAMP treatment of RAW cells will prove to be the murine ortholog of human ABC1. The protein does increase iodide transport in response to cAMP, however, and Becq *et al.* (26) have shown that protein kinase A phosphorylates it *in vitro*. The dependence of cholesterol efflux on PKA phosphorylation of this protein undoubtedly will be examined in short order. As there are other structural elements within the ABC1 protein that suggest interactions with different membrane-associated proteins, future studies also could show that these as yet unidentified proteins are responsible for the apoAI binding or cAMP regulation that Smith's lab has identified in the RAW cells.

The discovery of the ABC1 transporter's link to abnormal cholesterol efflux promises to lead to a host of new insights into lipid metabolism. Previous work implicating protein kinase C and phospholipases C and D activation in cholesterol efflux pathways now can be reconsidered in light of their effects on ABC1 (27, 28). The role of ABC1 in phospholipid efflux also can be explored. Recent work on a highly homologous ABC transporter, Rim, involved in phosphatidylethanolamine transport in rod photoreceptor outer segments, suggests that exploration will be a fruitful one (29). From a clinical standpoint, the inverse relationship between HDL levels and coronary artery disease makes the cholesterol efflux pathway and the ABC1 transporter potential targets for therapeutic agents designed to improve cholesterol removal from atherosclerotic plaques (30). Whether the many individuals with more modestly reduced HDL cholesterol levels and coronary heart disease also will have defects in the ABC1 transporter pathway is a question likely to engage the interest of epidemiologists and genecticists (31, 32). Finally, the enormous impact of ABC1 on the serum lipid profile, as evidenced by Tangier patients' dramatically reduced HDL and low density lipoprotein cholesterol levels, indicates that an understanding of this gene's function will profoundly affect our knowledge of serum lipoprotein metabolism.

It may be too much to assume that the spirit of Captain Smith, renowned for his irascibility, truculence, and conceit, is smiling at the work of the latest explorers to put Tangier Island again on the world's map. However, one suspects that a grudging respect for the genetic mapmakers would be forthcoming, as they, too, have helped open a new world whose further exploration is likely to continue to delight, inform, and confound us for many years to come.

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